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Purification of Human β_2-Adrenergic Receptor Expressed in Methylotrophic Yeast *Pichia pastoris*

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Human β_2-adrenergic receptor is a G-protein–coupled receptor with seven transmembrane helices, and is important in pharmaceutical targeting on pulmonary and cardiovascular diseases. N-terminal histidine-tagged gene constructs with optimized codon usage were designed so as to obtain *Pichia pastoris* transformants with a high expression level. The constructs were inserted into the pPIC9 vector, and then electroporated into the SMD1168 strains. The highest expression level obtained was about 4 mg/liter-culture broth. The dissociation constant of the receptor in the membrane fraction was 1.2 nM toward CGP-12177 antagonist. The receptor was solubilized with sucrose monolaurate and purified with a series of chromatography steps including anion-exchange, Ni-Sepharose, alprenolol-Agarose, and hydroxyapatite columns. The receptor was heterogeneously glycosylated, showing broad SDS-PAGE bands around 70–90 kDa. After endoglycosidase treatment, the receptor appeared as a single band around 45 kDa, and was further purified with hydroxyapatite and gel-filtration columns. The receptor was eluted as a sharp peak at the gel-filtration elution volume corresponding to a molecular mass of 117 kDa. The saccharide-trimmed receptor thus purified is homogeneous as analyzed with SDS-PAGE, shows the dissociation constant of 4.7 nM toward CGP-12177 antagonist, and is suitable for crystallization experiments.

Key words: β_2-adrenergic receptor, G-protein coupled receptor, membrane protein, *Pichia pastoris*, structural biology.

Abbreviations: β_2-AR, human β_2-adrenergic receptor; rβ_2-AR, histidine-tagged human β_2-adrenergic receptor; GPCR, G-protein–coupled receptor; K_d, dissociation constant; SML, sucrose monolaurate.

Human β_2-adrenergic receptor (β_2-AR) is an integral membrane glycoprotein with 413 amino-acid residues and molecular weight of 46,557 (*1*), and consists of N-terminal periplasmic, seven-transmembrane, and C-terminal cytosolic domains (*2*). β_2-AR belongs to the superfamily of G-protein–coupled receptors (GPCRs), and activates adenylate cyclase in response to agonist in the course of the activations of G-proteins, and increases the production of intracellular cAMP which induces various biological responses (*3*). β_2-AR is highly important in pharmaceutical targeting on pulmonary and cardiovascular diseases, and its structural information is essential for designing new drugs with higher potency and selectivity. However, the three-dimensional structural information on GPCRs is very limited: bovine rhodopsin of a photoreceptor is the only GPCR for which the crystal structure is available (*4*). The reasons for the limited structural information on GPCRs are mainly attributable to their difficulty in overexpression and in purification of solubilized active forms (reviewed in Ref. *5*).

To obtain β_2-AR in large quantities enough for crystallization experiments, we have used methylotrophic yeast *Pichia pastoris* for the expression of β_2-AR. In the *P. pastoris* expression, the post-translational modifications, such as *N*-glycosylation, would be expected to occur in a similar manor to mammalian cells. Cultivation of the cells needs much shorter time and lower cost than that of other eukaryotic cells, for example, baculovirus-infected cells. The expression (*6*) and purification (*7*) of the non-glycosylated form of β_2-AR in *P. pastoris* were reported, but obtainable amounts of the purified receptor were not quantified.

We report here, for the first time, the large-scale expression of *N*-glycosylated β_2-AR using *P. pastoris*, solubilization, and purification of saccharide-trimmed form of β_2-AR into a quantifiable amount.

MATERIALS AND METHODS

Recombinant β_2-AR Constructs—The DNA sequence of full-length β_2-AR was newly designed by replacing codons rarely used in *P. pastoris* with preferred ones according to data of Sreekrishna (*8*). The CTG codon for Leu, for example, which was preferred for human but rare for *P. pastoris*, was replaced with the TTG codon preferred for *P. pastoris*. All the codons in the designed DNA sequence were preferred for *P. pastoris*, as compared with the original β_2-AR DNA sequence in which 58% of the codons were preferred (*1*).

A histidine-tag sequence was attached to its N-terminus so as to make the purification easier. The histidine-tagged recombinant β_2-AR (rβ_2-AR) constructs were chemically synthesized, amplified by PCR reactions, and subcloned downstream of the α-factor signal sequence of the pPIC9 vector (Invitrogen, San Diego, CA). The pPIC9-rβ_2-AR vector thus prepared was linearized with *Sal*I and

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electroporated into *P. pastoris* strain SMD1168. Transformants were selected for their reversion of histidine-positive prototrophy. The genome DNAs of the selected transformants were isolated and analyzed by the dot-blot analysis (*9*) so as to confirm their incorporation of the rβ_2-AR DNA sequences. The selected transformants were stored as glycerol stocks at –70°C.

Fermentation of the Transformants—The glycerol stocks of the transformants of 100 μl were inoculated into 50 ml of BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6.0, 1.34% (w/v) yeast nitrogen base, 0.4 μg/ml biotin, and 1% (w/v) glycerol), and were pre-cultured for about 24 h at 30°C to a 600-nm optical density of 2 to 6. The cells were then inoculated into 1 liter of the BMGY medium in a 3-liter fermenter and cultured for 20 to 24 h until a cellular yield of wet cells increased to 180 to 200 mg/ml. A glycerol solution of 50% (w/v) was added to the culture broth when glycerol was completely consumed. The cell culture broth was kept at pH 6 by adding phosphoric acid or 25% (w/v) ammonia solution throughout the cultivation with the fermenter. Addition of the glycerol solution was stopped when the cellular yield of 180 to 200 mg/ml was achieved.

After glycerol was completely consumed, alprenolol antagonist was added to the culture broth to a final concentration of 3 μM, temperature was lowered to 25°C, and methanol was added to a final concentration of 0.15% (v/v) so as to induce the expression of rβ_2-AR. The cultivation was continued for 3 days by keeping the methanol concentration at 0.15% (v/v), which was controlled by alcohol-concentration controller DL-10A3 (ABLE Corporation, Tokyo, Japan).

Preparation of the Cell Membranes—Preparation of cell membranes as well as solubilization and purification of rβ_2-AR were performed at 4°C or on ice. Cells of 700 g wet weight were collected by centrifugation at 12,000 × *g* for 8 min, washed once with buffer A [75 mM sodium phosphate pH 7.4, 5% (w/v) glycerol, 1 mM phenylmethylsulfonyl fluoride], and resuspended in 3 liter of buffer A. Suspended cells were homogenized with glass beads in a stirrer. Cell debris were removed by centrifugation at 1,800 × *g* for 5 min, and a turbid supernatant solution containing cell membranes was obtained. Stock solutions of 50% (w/v) polyethylene glycol 8000 and of 5 M sodium chloride were added to the supernatant solution to a final concentration of 5% (w/v) and 200 mM, respectively. The resultant solution was stirred for 10 min. Precipitated membranes were collected by centrifugation at 17,000 × *g* for 10 min. The collected crude membrane fractions were frozen in liquefied nitrogen and stored at –30°C.

Solubilization and Purification of rβ_2-AR—Each of the membrane fractions was thawed and then suspended into 7 liter of buffer B (500 mM sodium chloride, 20 mM sodium phosphate pH 7.4, 1 mM phenylmethylsulfonyl fluoride). The suspended solution was mixed with 400 ml of a 20% (w/v) solution of sugar ester L-1695 (Mitsubishi-Kagaku Foods, Tokyo, Japan) containing 80% sucrose monolaurate (SML) and 20% sucrose laurate polyesters. After stirring for 15 min, non-solubilized fractions were removed by centrifugation at 17,000 × *g* for 10 min.

The supernatant solution containing solubilized rβ_2-AR as a micellar form with SML was mixed with 300 ml wet volume of anion-exchange resin Q-Sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) equilibrated with buffer C (500 mM sodium chloride, 20 mM Tris, pH 7.4) containing 1% (w/v) L-1695. After stirring for 15 min, the resin was removed by centrifugation at 12,000 × *g* for 10 min. The resultant supernatant solution was loaded onto a 200 ml Ni-Sepharose 6 Fast Flow column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), pre-equilibrated with buffer B containing 1% (w/v) L-1695. The column was washed with 500 ml of buffer B containing 1% (w/v) L-1695 and then with 600 ml of buffer D [500 mM sodium chloride, 50 mM Hepes pH 7.4, 0.1% (w/v) SM-1200 (Mitsubishi-kagaku Foods, Tokyo, Japan) containing 99% pure SML]. rβ_2-AR was eluted stepwise from the Ni-Sepharose column with buffer D containing 250 mM imidazole.

The eluted rβ_2-AR solution was loaded onto a 50 ml Q-Sepharose column pre-equilibrated with buffer C containing 0.1% SM-1200 so as to remove remaining phospholipids. The column was washed with 50 ml of buffer C containing 0.1% (w/v) SM-1200. Flow-through and wash solutions were mixed together, and galactose was added to a final concentration of 200 mM, so as to reduce non-specific adsorption of contaminants to sugar-based Agarose resin. The mixed solution was loaded at room temperature onto the alprenolol-Agarose column (*10*) of 250 ml pre-equilibrated with buffer D. The column with bound rβ_2-AR was cooled to 4°C where dissociation of rβ_2-AR from the resin was slow, washed with 600 ml of buffer D containing 200 mM galactose, and again with 600 ml of buffer D. The column was warmed to a room temperature, and rβ_2-AR was eluted overnight with 800 ml of buffer D containing 100 μM alprenolol.

To reduce the solution volume, the rβ_2-AR solution eluted from the alprenolol-Agarose column was loaded onto a 5 ml Ni-Sepharose column pre-equilibrated with buffer D. The column was washed with 10 ml of buffer D, and rβ_2-AR was eluted stepwise with 20 ml of buffer D containing 200 mM imidazole. Alprenolol was added to the eluted rβ_2-AR solution to a final concentration of 5 μM. The rβ_2-AR solution was frozen in liquefied nitrogen and stored at –30°C.

The frozen rβ_2-AR solution was thawed and its supernatant was obtained by centrifugation at 100,000 × *g* for 10 min. The supernatant was dialyzed overnight against a buffer containing 20 mM Hepes pH 7.4, 5 μM alprenolol, and 0.1% (w/v) SM-1200. Precipitates in the dialyzed solution were removed by centrifugation. Solutions of 5 M sodium chloride and of 1 M Hepes pH 7.4 were added to the dialyzed solution to final concentrations of 500 mM and 50 mM, respectively. The resultant solution was loaded onto the 5 ml Ni-Sepharose column pre-equilibrated with buffer D. The column was washed with 10 ml of buffer D containing 10 mM imidazole, and rβ_2-AR was eluted with a 60 ml solution of buffer D in a linear gradient from 10 mM to 200 mM imidazole. The eluted fractions exhibited an antagonist-binding activity and contained only glycosylated rβ_2-AR, as determined with SDS-PAGE analysis. The fractions were mixed together and dialyzed overnight against buffer E [10 mM sodium phosphate pH 7.4, 5 μM alprenolol, and 0.1% (w/v) SM-1200].

The dialyzed rβ_2-AR solution was loaded onto a hydroxyapatite CHT-II column of 5 ml (Bio-Rad, Hercules, CA)

which was pre-equilibrated with buffer E at a room temperature, and the column was washed with 15 ml of buffer E. rβ_2-AR was eluted with a 60-ml gradient of sodium phosphate solution, pH 7.4, starting from 10 mM and ending at 500 mM. rβ_2-AR fractions were pooled, and alprenolol was added to a final concentration of 5 μM.

To trim off *N*-linked oligosaccharides, endoglycosidase H (New England Biolabs, Ipswich, MA) was added to the rβ_2-AR solution at 30 units per microgram of rβ_2-AR. The rβ_2-AR solution was incubated at 20°C for 30 min, and dialyzed overnight against buffer E at 4°C. The endoglycosidase-treated rβ_2-AR was purified again with the hydroxyapatite column, and further dialyzed overnight against buffer F of 100 mM sodium chloride, 20 mM Hepes pH 7.4 and 0.1% (w/v) SM-1200. The dialyzed rβ_2-AR solution was concentrated by Centricon-100 (Millipore, Billerica, MA), and applied to a Superdex 200 16/10 gel-filtration column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) pre-equilibrated with buffer F at the flow speed of 0.5 ml per min. The molecular weight of micellar rβ_2-AR was estimated from the calibrated elution time using the kit of Molecular Weight Markers for Gel-filtration Chromatography (Sigma-Aldrich, St. Louis, MO).

Antagonist-Binding Assay—The membrane-suspended solution containing rβ_2-AR was diluted to 0.1–0.5 nM with buffer G of 75 mM Tris pH 7.4, 12.5 mM magnesium chloride, and 2 mM EDTA, containing 4% (v/v) protease inhibitor cocktail solution (Roche, Basel, Switzerland). The diluted membrane suspension of 200 μl was mixed with 50 μl of buffer G containing 25 nM radio-labeled antagonist [5,7-^{3}H]-(–)CGP-12177 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and 0.1 mg/ml bovine serum albumin. The mixture was incubated at 37°C for 1 h. Complexes with radio-labeled CGP-12177 were collected by vacuum filtration with Whatman GF/C filters (Brentford, UK). The filters were quickly washed three times with 1.5 ml of cold buffer containing 25 mM Tris pH7.4 and 1 mM magnesium chloride. Radioactivity levels on the filters were measured by liquid-scintillation counting. Non-specific binding to the ligand was determined in the presence of 1 mM alprenolol. The number of rβ_2-AR molecules remaining on the filter was calculated using the number of the specifically bound radio-labeled CGP-12177 and the dissociation constant (K_d) value toward CGP-12177.

Each of the rβ_2-AR solutions was diluted to 1.0–0.2 nM with buffer H of 100 mM sodium chloride, 50 mM Tris pH 7.4, 0.1% (w/v) SM-1200, and 0.1 mg/ml bovine serum albumin. The diluted rβ_2-AR solution of 50 μl was applied to a microbiospin-6 gel-filtration column (Bio-Rad, Hercules, CA) pre-equilibrated with buffer H. The flow-through solution was mixed with 12.5 μl of buffer H containing 25 nM of radio-labeled CGP-12177. After incubation at 25°C for 1 h, the mixture was applied to the microbiospin-6 column so as to remove unbound radio-labeled CGP-12177. Radioactivity levels of the flow-through solution were measured by liquid-scintillation counting, and the number of solubilized rβ_2-AR molecules in the flow-through solution was calculated as described above.

The dissociation constant was derived through binding assays, by incubating the rβ_2-AR solution in 15–0.25 nM concentrations of the radio-labeled CGP-12177. The number of radio-labeled CGP-12177 specifically bound to rβ_2-AR was determined as described above. Data analyses were performed on GraFit software (Erithacus Software, Surrey, UK), and both the K_d value and the concentration of rβ_2-AR capable of binding to CGP-12177 were determined simultaneously.

N-Terminal Sequencing and Estimation of Protein Concentration—Solubilized and saccharide-trimmed protein samples were electrophoretically migrated onto SDS-PAGE, and protein bands were transferred to PVDF membranes. Protein bands were analyzed with an automated Edman degradation sequencer of Procise 491HT (Applied Biosystems, Foster City, CA). Protein concentrations were determined with the Bio-Rad Protein Assay Kit using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Expression levels of rβ_2-AR in the membrane fraction of *P. pastoris* increased linearly after the addition of methanol to the culture media. Three days after the methanol induction, the expression level reached to the maximum value of about 4 mg β_2-AR per liter of the culture broth, and continuous addition of methanol approximately doubled the volume of the culture broth. From then on, cells grew slowly, but the expression level per wet weight of cells decreased.

Because the rβ_2-AR purity was quite low in early stages of the purification, rβ_2-AR was detected only with the antagonist-binding assay. rβ_2-AR became identifiable, on the SDS-PAGE stained with Coomassie Brilliant Blue R-250, after the alprenolol-Agarose purification and adsorptive concentration on the Ni-Sepharose column. Table 1 shows yields of rβ_2-AR in the course of the purification.

The rβ_2-AR purified with the alprenolol-Agarose column was concentrated and dialyzed against the low-ionic-strength buffer of 20 mM Hepes pH 7.4, 5 mM alprenolol, and 0.1% SML. During dialysis, about two thirds of the total protein were precipitated, but rβ_2-AR remained soluble (Table 1). The precipitates containing impurities were easily removed by centrifugation, resulting in doubling the specific activity. This purification step was necessary prior to the clear separation of two forms of rβ_2-AR with Ni-Sepharose column chromatography, as is described later on.

The dialyzed rβ_2-AR solution was applied to the Ni-Sepharose column, and eluted with the linear-gradient in the imidazole concentration. The elution profile from the Ni-Sepharose column and the resultant SDS-PAGE gel are shown in Fig. 1. The elution profile indicates that two forms, A and B, of rβ_2-AR are present and both capable of ligand binding. Form A was eluted as a broader peak later than fraction 6 in Fig. 1. Form B was eluted as a sharp peak at fractions 5 and 6, and appeared as a narrow band at around 43 kDa in the SDS-PAGE. Form A further purified with the hydroxyapatite column appeared as broad SDS-PAGE bands around 70–90 kDa and as a single band around 45 kDa after the endoglycosidase H treatment, as shown in Fig. 2. This difference in the molecular masses indicates that form A is highly and heterogeneously glycosylated. Endoglycosidase H uniformly cleaves off saccharide moieties from *N*-glycosylated proteins expressed in *P. pastoris* (*11*), leaving a single hydrophilic *N*-acetyl glucosamine at the glycosylated asparagine residue. This

Table 1. **Purification steps of recombinant human β_2-adrenergic receptor.**

Step	rβ_2-AR*[1] (pmol)	Total protein*[2] (mg)	Yield (%)	Specific activity (pmol rβ_2-AR/mg protein)
Crude membrane	320,000	53,000	100	6.0
Solubilization by SML*[3]	290,000	49,000	91	5.9
Ni-Sepharose (first)	110,000	4,400	34	25
Alprenolol-Agarose column	54,000	25	17	2,200
Dialysis	39,000	8.6	12	4,500
Ni-Sepharose (second)	25,000	4.0	7.8	6,300
Dialysis	18,000	3.1	5.6	5,800
Hydroxyapatite (first)	4,300	0.90	1.3	4,800
Hydroxyapatite (second, after glycosidase treatment)	2,200	0.25	0.7	8,800
Gel filtration	1,700	0.16	0.5	11,000

*[1]Total mol number of rβ_2-AR was calculated as described in Materials and Methods. 21,479 pmol corresponds to 1 mg rβ_2-AR. *[2]Determined with the Bradford method using bovine serum albumin as a standard. *[3]SML stands for sucrose monolaurate detergent.

trimming does not decrease the solubility and leads to successful crystallization and structure determination (*12*). In the case of rβ_2-AR form A, the saccharide moieties are cleaved off uniformly by endoglycosidase H as indicated by the narrow band in the SDS-PAGE, and the decrease of the solubility is not observed after the endoglycosidase treatment. The saccharide-trimming is thus intended for the successful crystallization.

N-terminal sequencing of form A revealed that its first nine N-terminal amino-acid residues were six histidines and Met1-Gly2-Gln3, indicating that the α-factor signal sequence was cleaved off properly by internal KEX2 endopeptidase of *P. pastoris*.

N-terminal sequencing of form B revealed that first five N-terminal amino-acid residues are Ala13-Pro14-Asn15-Arg16-Ser17, indicating that the α-factor signal sequence, histidine-tag, and 12 N-terminal-side residues from Met1 to Leu12 were cleaved off. β_2-AR has three *N*-glycosylation sites, Asn6, Asn15, and Asn187 (*13*). N-terminal sequencing of form B clearly indicated that Asn15 is not glycosylated. The narrow band of form B in the SDS-PAGE suggests that Asn187 is also not glycosylated, since glycosylation in *P. pastoris* is often highly heterogeneous. Neither non-glycosylated form A nor glycosylated form B was detected with SDS-PAGE and N-terminal sequencing. Form B therefore was probably expressed in *P. pastoris* cells through a different post-translational pathway from that of form A. It is not clear whether the cleavage between Leu12 and Ala13 prevented the glycosylation of rβ_2-AR or conversely non-glycosylated rβ_2-AR underwent the cleavage. Form B lacking histidine-tag was bound to Ni-Sepharose resin. It is reported that β_2-AR has a zinc-binding site at the cytoplasmic extension of the transmembrane helices 5 and 6 (*14*). The zinc-binding site supposedly gave rise to affinity toward divalent nickel ions.

The expression of highly glycosylated form A is in contrast to the result reporting that only non-glycosylated rβ_2-AR was expressed in *P. pastoris* (*6*, *7*). Oligosaccharide moieties of β_2-AR are reported to have a functional importance; prerequisite for the down regulation of the β_2-AR cellular signaling (*13*). Glycosylated rβ_2-AR reported here is certainly suitable for the structural study for elucidation of the signaling mechanism.

The yield of rβ_2-AR in the second Ni-Sepharose step is about 70% and is higher than the yield of 40% in the first step in which the total protein was reduced to 9%. The yield of rβ_2-AR in the first hydroxyapatite step is around 25% and is lower than that of the Ni-Sepharose steps. Even after the endoglycosidase treatment, the second hydroxyapatite step with the improved recovery of 50% doubled the specific activity. Although unrecovered proteins which were tightly adsorbed on the columns are noticed for each step, the higher yields at the latter steps indicate the progressive purification.

In the final stage of the purification, rβ_2-AR form A were eluted as a single peak from the gel-filtration column, as shown in Fig. 3. The elution time of the peak corresponds to a molecular mass of 117 kDa, and the absence and the presence of the alprenolol antagonist did not alter the elution time of rβ_2-AR. The elution time of micellar SML gave a molecular mass of 80–90 kDa in the same condition. If rβ_2-AR exists as dimeric aggregates in complex with micellar SML, forms A and B supposedly tend to associate together and are difficult to separate them. However, they were easily separated with the Ni-Sepharose column. Hence, the 117-kDa mass, nearly equal to the sum of the masses of monomeric rβ_2-AR and micellar SML, corresponds to the monomeric rβ_2-AR in detergent complex with SML. The large detergent complex of turkey β_1-AR solubilized with dodecyl-β-D-maltoside was also reported to be 120 kDa (*15*). SML is suitable for purification of rβ_2-AR since it efficiently solubilizes rβ_2-AR without loss of activity. The excessively large amount of SML associated with rβ_2-AR might interfere in protein–protein interactions prerequisite to the crystallization.

The dissociation constant K_d for the membrane fraction of rβ_2-AR to antagonist CGP-12177, which consists of heterogeneously glycosylated form A and homogeneous form B, is 1.2 nM as shown in Fig. 4. The endoglycosidase treatment did not substantially affect ligand-binding affinity: the K_d values of purified and saccharide-trimmed rβ_2-AR form A for CGP-12177 was 4.7 nM, as shown in Fig. 4. Although form B is partly purified, its K_d value is also determined to be 4.7 nM. These suggest that not only the glycosylation but also the removal of the N-terminal 12-residues virtually do not affect the binding activity of solubilized rβ_2-AR, and that the K_d values of these two forms in the membrane are virtually identical. The observed K_d value is comparable to the K_d value of 1.2 nM reported for β_2-AR expressed in human fat cells

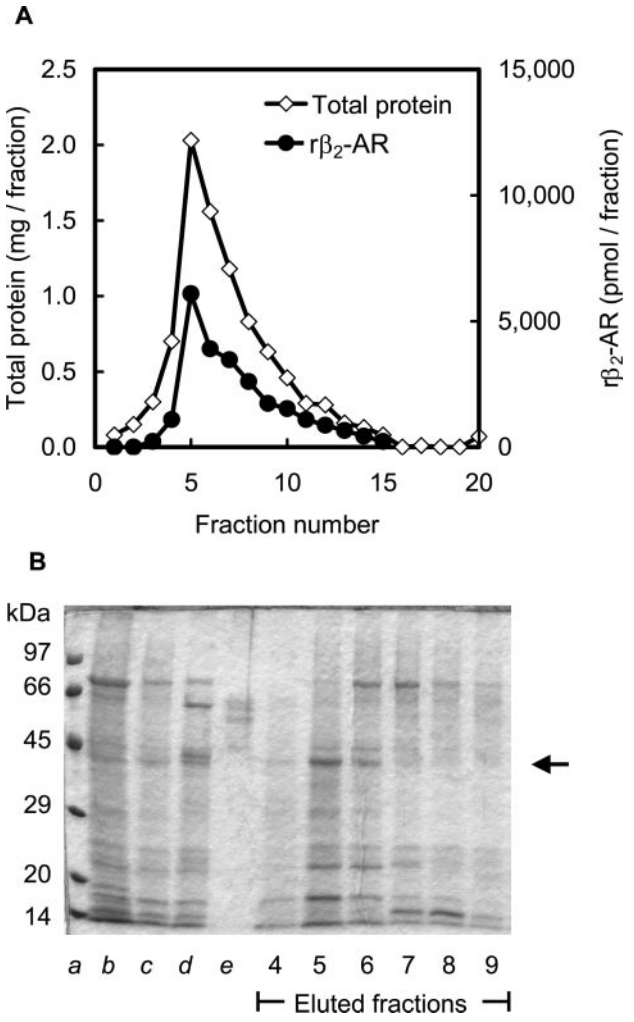

Fig. 1. **Purification of rβ_2-AR with the first Ni-Sepharose column.** Panel A, elution profile. Panel B, SDS-PAGE analysis of fractions eluted from the Ni-Sepharose column. The gel was stained with Coomassie Brilliant Blue R-250. Lane a, molecular weight markers. Lane b, rβ_2-AR eluted from the alprenolol-Agarose column. Lane c, dialyzed rβ_2-AR of b. Lane d, dialyzed rβ_2-AR treated with endoglycosidase H_f (New England Biolabs, Ipswich, MA). The upper band emerging at around 45 kDa as compared to lane c is oligosaccharide-trimmed form A, and the lower band indicated with an arrow is non-glycosylated form B. The band around 60 kDa is endoglycosidase H_f. Lane e, column flow through fraction. Numbered lanes, fractions 4–9 from the Ni-Sepharose column.

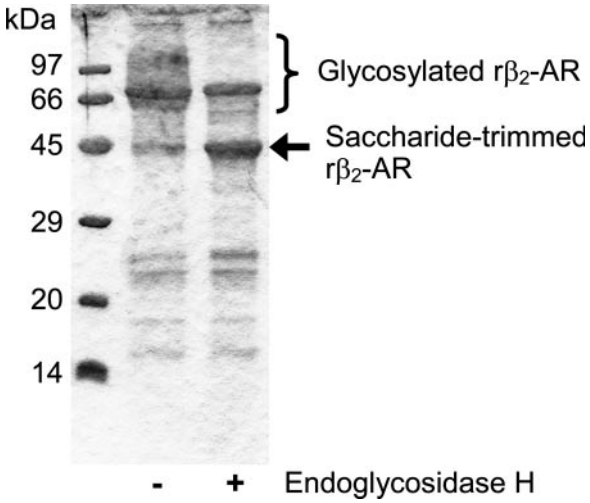

Fig. 2. **Effect of the endoglycosidase-treatment of rβ_2-AR.** The SDS-PAGE gel was stained by Coomassie Brilliant Blue R-250. The lane marked with the minus symbol, glycosylated form A purified with the hydroxyapatite column; the lane with the plus, after the treatment with endoglycosidase H. The dense and narrow band around 70 kDa is an unknown protein which was removed completely with the subsequent purification steps.

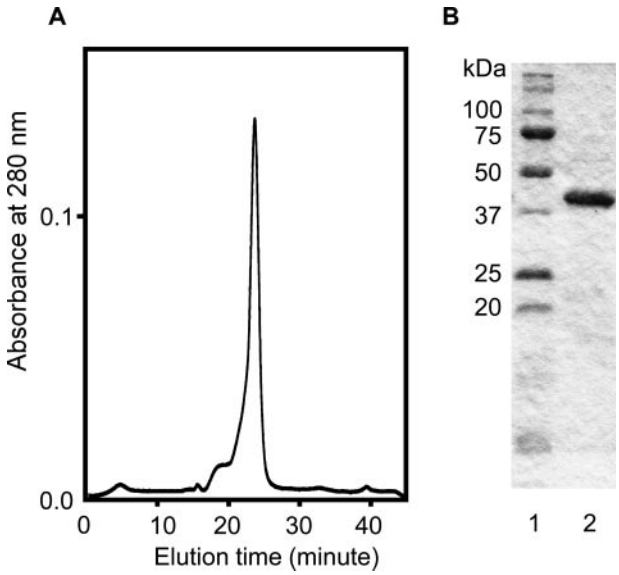

Fig. 3. **Gel-filtration purification of rβ_2-AR.** Panel A, rβ_2-AR eluted from the Superdex 200 gel-filtration column. Panel B, SDS-PAGE gel stained by Coomassie Brilliant Blue R-250. Lane 1, markers; lane 2, purified rβ_2-AR.

(*16*). The high affinity of rβ_2-AR to the antagonist indicates the properly folded rβ_2-AR structure as in the case of β_2-AR expressed in the human fat cells.

The specific activity of rβ_2-AR purified with the gel-filtration is obtained as 11,000 pmol rβ_2-AR per milligram total protein. This value is about a half of the theoretical maximum of 21,479 pmol β_2-AR per milligram protein, although little amount of proteins was contaminated as analyzed with SDS-PAGE (Fig. 3) There were several factors that would make the apparent specific activity lower. When rβ_2-AR concentration was estimated with the molar extinction coefficient of 65,610 at 280 nm, which is derived from the amino-acid composition of rβ_2-AR (*17*), the protein concentration is about 80% of that determined with the assay kit. Expected recovery of rβ_2-AR from the spin-column used in the assay was 94%. Taking these into account, the specific activity of the purified rβ_2-AR becomes about 70% of the theoretical maximum. It is known that some co-factors, lipids bound to protein for example, affect the binding properties of recombinant GPCRs (*18*, *19*). The loss of such co-factors during solubilization and purification would lower the specific activity of the otherwise fully active rβ_2-AR, and might also explain the somewhat lower affinity of solubilized rβ_2-AR toward antagonist CGP-12177 as compared to that of rβ_2-AR in the membrane fraction.

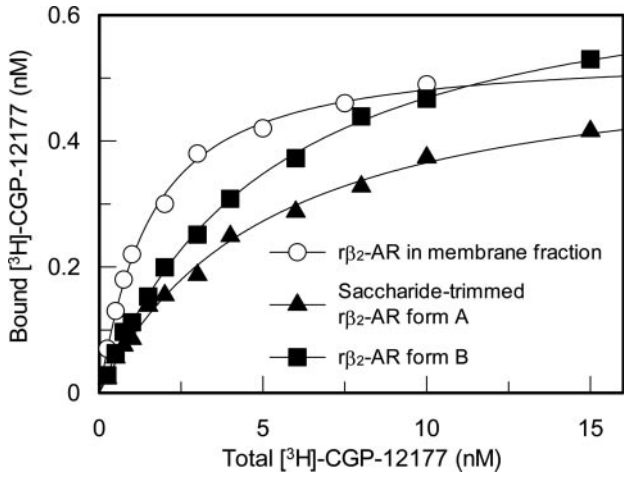

Fig. 4. **Concentration-dependence curves for specific binding of antagonist CGP-12177 to rβ$_2$-AR.** The dissociation constant K_d, concentration of rβ$_2$-AR capable of binding to antagonist [rβ$_2$-AR], and their standard errors were calculated by non-linear least-squares fitting: K_d = 1.2 ± 0.1 nM and [rβ$_2$-AR] = 0.54 ± 0.01 nM for rβ$_2$-AR in the membrane fraction, K_d = 4.7 ± 0.4 nM and [rβ$_2$-AR] = 0.54 ± 0.02 nM for the saccharide-trimmed form A, and K_d = 4.7 ± 0.2 nM and [rβ$_2$-AR] = 0.70 ± 0.01 nM for the partially-purified form B.

The purified rβ$_2$-AR was stable in the presence of the antagonist at 4°C; about 70% of the antagonist-binding activity of purified rβ$_2$-AR in buffer F containing 5 μM alprenolol remained after the storage for 1 week. The yield of rβ$_2$-AR capable of binding to antagonist is about 80 μg per liter of BMGY medium. Scaling up the cultivation of *P. pastoris* as well as practicing the purification method itself would yield purified rβ$_2$-AR in a quantity sufficient for structural study. At the present, about 0.9 mg of purified rβ$_2$-AR are routinely obtained from a 10-liter scale cultivation. Purified rβ$_2$-AR is concentrated higher than 4 mg/ml. When the recent technologies reporting the nanoliter- and even picoliter-scale crystallization of membrane proteins (*20*) is utilized, tens of thousands of trial can be performed.

In conclusion, we have expressed glycosylated rβ$_2$-AR in *P. pastoris* with the antagonist-binding activity comparable to that of β$_2$-AR in the human cells, and developed the purification procedure of solubilized and saccharide-trimmed rβ$_2$-AR that is homogenous as analyzed with SDS-PAGE. The purified rβ$_2$-AR, alternatively one exchanged with detergents other than SML, hopefully suffices for crystallization attempts.

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